our series. Still, 11.5% of patients have no mutation after extensive analysis of the *OCA1–4*, *OCA6*, *OA1*, and *HPS1* genes, thus suggesting that other genes involved in OCA still remain to be identified.

Written informed consent was received from the patients. The authors adhere to the Declaration of Helsinki Principles. Experiments were approved by the Comité de protection des Personnes Bordeaux—Outre Mer III.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We wish to thank the French Albinism Association Genespoir, the Union Nationale des Aveugles et Déficients Visuels, and the Ministry of Health (France) for their financial support. FM-P had a PhD studentship from the Fondation pour la Recherche Médicale (France). DNA sequencing was performed at the Genome Transcriptome Platform of Bordeaux (grants from the Conseil Régional d'Aquitaine nos 20030304002FA and 20040305003FA and from the European Union, FEDER no. 2003227). BPL is a senior clinical investigator of the Research Foundation Flanders (FWO).

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Long-Term Survival of Type XVII Collagen Revertant Cells in an Animal Model of Revertant Cell Therapy

Journal of Investigative Dermatology (2014) **134,** 571–574; doi:10.1038/jid.2013.308; published online 15 August 2013

TO THE EDITOR

Revertant mosaicism is the coexistence of mutant cells carrying germline mutations and revertant cells that have spontaneously corrected the germline mutation by a somatic reverse mutation. Revertant mosaicism has been reported for a number of genetic diseases (Pasmooij and Jonkman, 2012), including epidermolysis bullosa. Moreover, the first case of revertant mosaicism in skin was found in a Dutch patient 026-01 with junctional epidermolysis bullosa caused by mutations in *COL17A1*. The patient was compound heterozygous for a maternal deletion in exon 18, c.1601delA, and paternal nonsense mutation in exon 51, c.3676C>T (Jonkman *et al*, 1997). Owing to gene conversion, the c.1601delA mutation was corrected and the patient presented a clinically healthy skin patch on her forearm (Figure 1a), where affected (mutant) and corrected (revertant) keratinocytes coexisted (Figure 1b). Recently, we found revertant mosaicism to occur in all Dutch patients with junctional epidermolysis bullosa (Jonkman and Pasmooij, 2009; Pasmooij *et al*, 2012).

Naturally corrected keratinocytes expressing type XVII collagen (C17) harvested from a revertant patch can be used for autologous cell therapy.

Abbreviation: C17, type XVII collagen

Accepted article preview online 24 July 2013; published online 15 August 2013

Unfortunately, the first attempt to transplant revertant keratinocytes did not succeed because of a surprisingly low percentage of revertant cells in the graft (3%) (Gostynski *et al*, 2009). To explain the depletion of revertant cells, we have analyzed the process of skin equivalent production. Additionally, we have decided to study the feasibility of revertant cell therapy in an animal model to assess the long-term survival of revertant cells after engraftment.

Written, informed consent was obtained, and all procedures were conducted in accordance with the Declaration of Helsinki principles. Our revertant cell source was the revertant patch on the forearm of patient 026-01 (Figure 1a). A previous biopsy specimen from this area stained for C17 showed 50% revertant cells (Figure 1b; Jonkman *et al*, 1997; Pasmooij *et al*, 2005, 2012). Next to the earlier diagnostic biopsy location, a 6-mm punch biopsy was taken (Figure 1c), from which keratinocytes and fibroblasts were isolated with collagenase and trypsine/EDTA enzymatic digestion and cultured as described before (Llames *et al*, 2006). Keratinocytes were cultured on a feeder layer and passaged when confluent to expand the culture for animal model experiments and banking. The first passage was performed 9 days after isolation and the second passage (p2) was done 7 days later. During both passages a side sample was taken, cultured on coverslips, airfixed, and the number of revertant cells was assessed by immunofluorescence (IF) microscopy with the 1A8C mAb against C17 (gift from Dr K. Owaribe, Nagoya, Japan). Stainings showed 40% revertant cells after first passage, and 25% revertant cells after the second passage (Figure 1e and i). A sample of the keratinocytes was further cultured, which led to a drop of revertant cells to 15%, 1%, and $<1\%$ after passage 3, 4, and 5, respectively. Growth potential was tested using colony-forming efficiency assay, which showed that cells isolated from the revertant patch had growth potential comparable to keratinocytes from a healthy patient (Supplementary 1 online). Colonies were further stained with VK4 mAb, which revealed that the colony-forming potential was higher in revertant cells (39% of colonies were revertant compared with 15% of revertant cells in the population used for the assay), but their division rate, represented by colony size, was lower (Figure 1d).

From the cultured patient's fibroblasts and p2 keratinocytes, a 75-cm^2 bioengineered skin equivalent was produced as described before (Llames *et al*, 2004, 2006). Briefly, a plasma-based scaffold filled with fibroblasts was used as a dermal component of the bioengineered revertant skin: 7.5×10^4 cultured fibroblasts were resuspended in 10 ml of donor plasma (obtained from the blood bank), 10 mg of tranexamic acid (Amchafibrin, Rotapharm, Barcelona, Spain), and 2 ml of $CaCl_2$ 1%, and adjusted to 25 ml by adding NaCl 0.9%. The mixture was placed in a 75-cm^2 tissue culture flask and allowed to solidify at 37 °C for 30 minutes. Once the dermal equivalent solidified, it was covered with culture medium and 24 h later cultured keratinocytes were seeded. After 8 days, when keratinocytes reached confluence, the skin equivalent was harvested and moved to the animal facility. The equivalent was then divided into four pieces, 9 cm^2 each, for grafting, and samples were frozen for the C17 immunofluorescence analysis. The sample sections were stained with 1A8C mAb against C17 and analyzed by the measurement of C17-positive fragments. This analysis revealed that 20% of the graft consisted of revertant cells (Figure 1f).

Four immunodeficient nu/nu mice were transplanted as described before (Llames *et al* 2004; Garcia *et al*, 2007). Biopsies from the engrafted skin (Figure 1g) were taken after 10 and 16 weeks and directly frozen in liquid nitrogen. Each of the four biopsies was cut into 4-μm sections and stained with 1A8C or VK4 mAb (Dr HH Pas, Groningen, The Netherlands) (Yuen, 2012) against C17 to assess revertant cell survival, and with mAb (AF109) specific for murine keratin 1 (Prof D Roop, Denver, Colorado, USA) (Yuspa *et al*, 1989) to distinguish human and murine epidermis. Once again, the length of C17-positive fragments and the total length were measured and analysis revealed that revertant cells were present in 20% area of the human epidermis after both time points (Figure 1h and i). This corresponded with the percentage of revertant cells found in the skin equivalent and suggested a long-term survival of C17-producing cells. To prove that the reversion mechanism was present in the areas positive for C17, laser dissection microscopy followed by nested PCR was performed on all biopsies as described earlier (Pasmooij *et al*, 2005). In fragments of human epidermis positive for C17, we found the same post-zygotic reversion mutation as in donor site keratinocytes on the patient's forearm, i.e. the loss by gene conversion of the maternal *COL17A1*: c.1601delA mutation.

The reason for a decreased percentage of revertant cells remains unknown. It has been shown that C17 influences hair follicle stem cells in mice and that cells lacking C17 have a different phenotype *in vitro* (Hamill *et al*, 2011). Furthermore, C17 deficiency activates a proinflammatory pathway by upregulation of NF-κB, whereas in healthy keratinocytes that express C17, levels of NF-κB are low (Van den Bergh *et al*, 2012). Therefore, the growth advantage in cultures of C17-negative keratinocytes over C17-positive keratinocytes might be explained by NF-κB activation that stimulates epidermal proliferation in the C17-negative cells (Duheron *et al*, 2011). Further studies are required to assess whether such differences account for a growth advantage of mutant keratinocytes. To overcome the *in vitro* depletion of revertant cells for a therapeutic approach, a method for selection of C17 revertant cells compatible with clinical patient care is needed.

In this study we show that, after a marked decrease during *in vitro* expansion on plastic, the percentage of revertant keratinocytes stabilizes during skin equivalent production and remains stable *in vivo* (Figure 1i). This proves the long-term survival of revertant keratinocytes, suggesting that we transplanted revertant epidermal stem cells. Hereby, we demonstrate the feasibility of the revertant cell therapy for C17-deficient patients.

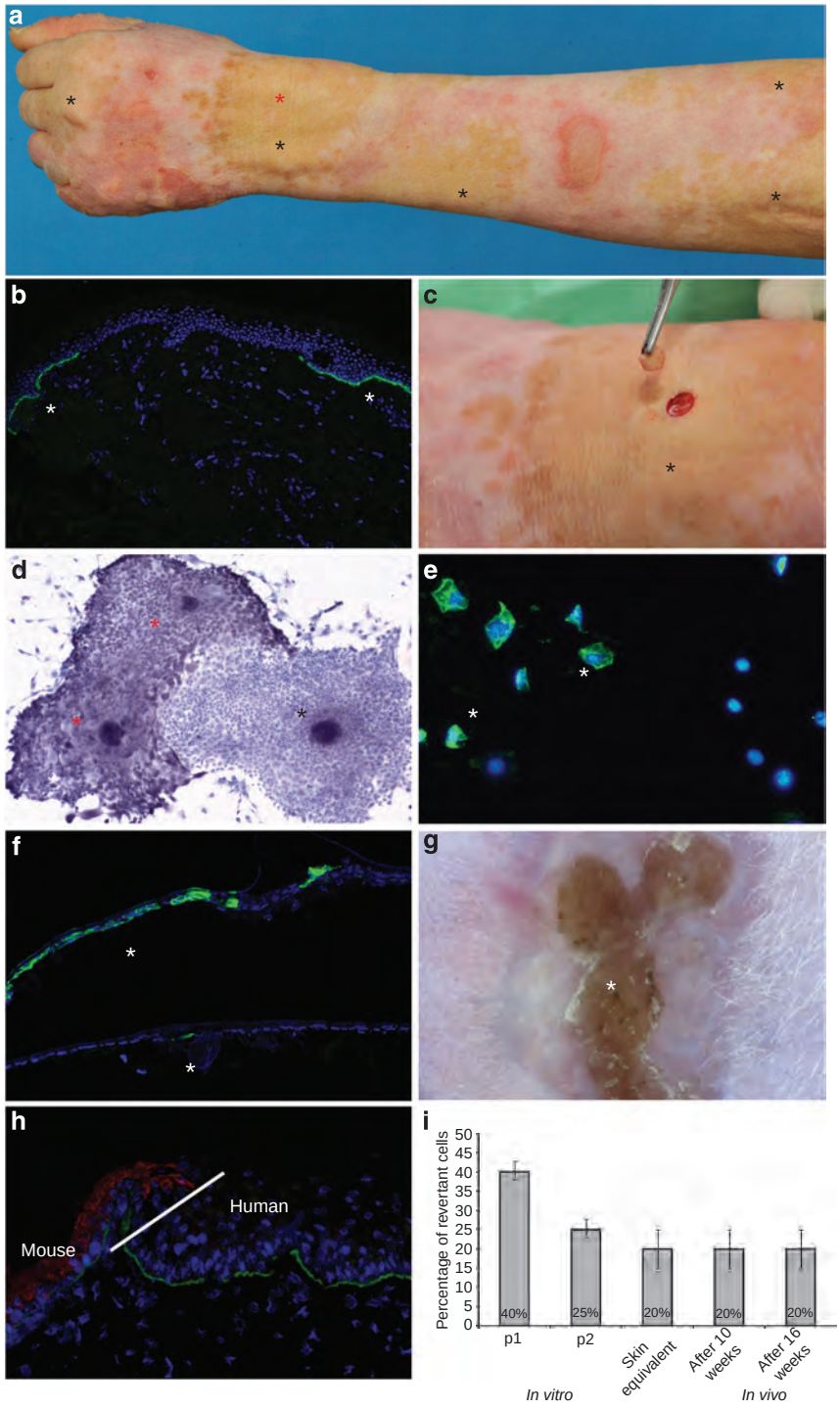

Figure 1. Long-term survival of C17 revertant cells in a skin-humanized mouse model. (**a**) Patient 026-01: left arm, outlined revertant patches with black asterisks. The red asterisk marks the location of the previous diagnostic biopsy specimen. (**b**) Immunofluorescence of the diagnostic biopsy showing 50% of the cells being revertant (white asterisks). (**c**) Biopsy for cell culture is taken from the revertant patch (outlined) next to the previous diagnostic biopsy location (red asterisk). (**d**) Immunohistochemical staining of the colony-forming efficiency assay with VK4 mAb showing two small revertant colonies (red asterisks) next to a large mutant colony (black asterisk). (**e**) Cultured keratinocytes on coverslips stained with 1A8C mAb (green) against the intracellular domain of C17. In blue cell nuclei. Revertant keratinocytes are marked with white asterisks. (**f**) Samples of skin equivalent stained with 1A8C mAb (green). White asterisks indicate the revertant regions of the epidermis. (**g**) Revertant graft (white asterisk) on mouse back 6 weeks after transplantation. Pigmentation is visible where human skin is present. (**h**) Graft on mouse sampled 10 weeks after transplantation and stained with VK4 mAb (green) against the intracellular domain of C17 and with mAb against mouse keratin 1 (red). In blue cell nuclei. The white line separates murine (Mouse) and human (Human) epidermis. (**i**) Percentage of revertant cells during *in vitro* culture, production of the skin equivalent, and after transplantation. For *in vitro* analysis after passage 1 and 2, 4,761 and 2,924 cells were counted, respectively.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by the Priority Medicines Rare Diseases (E-RARE) grant 113301091 from the Netherlands Organisation for Health Research and Development (ZonMw). MFJ is supported by a grant from Vlinderkind (Dutch Butterfly Child Foundation). FL is supported by grants PI11/0125 from Spanish ISCIII and P2010BMD-2359 from Comunidad de Madrid. MDR is supported by grants SAF 2010-16976 from MICINN and S2010/BMD-2420 (CELLCAM) from Comunidad de Madrid.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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NKp46-Specific Expression on Skin-Resident CD4+ Lymphocytes in Mycosis Fungoides and Sézary Syndrome

Journal of Investigative Dermatology (2014) **134,** 574–578; doi:10.1038/jid.2013.321; published online 29 August 2013

TO THE EDITOR

Sézary syndrome (SS), mycosis fungoides (MF), and transformed mycosis fungoides (TMF; Olsen *et al.*, 2007) belong to the heterogeneous group of cutaneous T-cell lymphoma (CTCL). They are characterized as extranodal non-Hodgkin lymphoma from malignant, mature T lymphocytes that home to the skin and persist there (Girardi *et al.*, 2004). SS and MF are the most common CTCL types. Recent genetic and phenotypic studies suggest that MF and SS, previously considered as different stages of the same disease, arise from two distinct T-cell subsets (van Doorn *et al.*, 2009; Campbell *et al.*, 2010). Pathological diagnosis is often hampered by the small proportion of malignant cells and by the morphological similarity to inflammatory skin diseases (ISDs). Thus, a reliable cutaneous biomarker able to distinguish malignant cells from reactive benign T cells in the infiltrate of CTCL is still needed. Most previous studies have focused on blood malignant T lymphocytes in patients with leukemic disease (Vonderheid *et al.*, 2001; Ferenczi *et al.*, 2002; Su *et al.*, 2003; Bensussan *et al.*, 2011). When skin samples were considered, a specific expression of *EphA4*, *Twist*, *TOX*, *PDCD1*, or *CDK158k/KIR3DL2* gene was found in MF or SS patients.

NKp46 has been shown on blood malignant CD4+ T lymphocytes in SS but not in MF or in TMF (Bensussan *et al.*, 2011). NKp46 belongs to natural

Abbreviations: CBCL, cutaneous B-cell lymphoma; CTCL, cutaneous T-cell lymphoma; ISD, inflammatory skin disease; LM, laser microdissection; NCR, natural cytotoxicity receptor; RT-PCR, real-time PCR; SEM, standard error of the mean; SS, Sézary syndrome; MF, mycosis fungoides; TMF, transformed mycosis fungoides

Accepted article preview online 30 July 2013; published online 29 August 2013